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DRUG DEVELOPMENT AGAINST VIRAL DISEASES

ANNUAL REPORT

GREGORY H. TIGNOR, SC.D.

1 FEBRUARY 1987

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Seventy-three compounds were tested for toxicity and/or antiviral activity against Congo-Crimean hemorrhagic fever (CCHF) virus in infant mice. Most compounds were tested using a single dose of drug administered 45 minutes before virus. Nine drugs were also tested after multiple doses of drug. Data were analyzed for protective effect (PE) and geometric mean time to death (VR). PE and VR correlated so that the VR will serve in the future as the sole measure of drug efficacy. Variation in the geometric mean time to death for mock-treated control (VC) and positive drug control animals (VR+) was determined. Standard errors for VC were 0.66 days for single dose experiments and 0.73 days for multiple dose experiments. Variation about the values for the positive control was lower with SE of 0.13 and 0.14 days for single and multiple dose experiments, respectively.

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SUMMARY

Seventy five compounds have been tested for toxicity and/or antiviral activity against Congo-Crimean hemorrhagic fever virus (CCHF) in infant mice. Most compounds were tested using a single dose of drug administered 45 minutes before virus. Nine drugs were also tested after multiple doses of drug. Data were analyzed in two ways: (1) Protective effect (PE) as determined by the ability of drug treatment to reduce mortality as compared to mock-treated animals. (2) Geometric mean time to death (VR) as determined by a ratio of the geometric mean time to death for drug-treated animals to the geometric mean time to death for mock-treated animals (VC). Our analysis showed that the two values correlate sufficiently such that the VR will serve as our sole measure of efficacy in future experiments. Variation within the test system was measured by determining variation in the geometric mean time to death for both control (mock-treated animals, VC) and for animals protected by one effective drug (i.e., a positive control, VR+). Standard errors for VC values were 0.66 days for single dose experiments and 0.73 days for multiple dose experiments. Variation about the values for the positive control was lower with standard errors of 0.13 and 0.14 days for single and multiple dose experiments, respectively.

Two drugs have shown reproducible protection from mortality and reproducible prolongation of survival time in primary testing against CCHF virus. These drugs include AVS#1 which is most effective. It has been adopted as the positive control in all tests. VR+ values ranged from 1.85 to 2.39 after a single dose of drug. After multiple injections of drug, the VR+ values ranged from 2.68 to 2.88. AVS#253, while less dramatic, reduced mortality by 1.4 logs after a single dose, and 1.3 logs after multiple doses. VR's are 1.44 and 1.68 respectively.

Other drugs have shown VR values approaching that of AVS#1 in single tests; these drugs warrant additional testing to determine reproducibility. Drugs of interest include AVS#'s 71, 78, 1970, 2137, and 2140. Each of these drugs had a VR of 2.39.

Drug AVS#1 was tested in primates (Saimiri sciureus) using two different schedules of drug administration. In this first experiment, drug was given at the time of and after subcutaneous infection with yellow fever virus. The only observed protective indices were a prolonged survival time and a slightly lowered peak viremia titer. In a second experiment, animals were given drug beginning three days prior to subcutaneous virus infection and continuing for 11 days thereafter. Mortality was reduced by 40% in drug-treated animals and the geometric mean survival time was significantly increased from 6.14 in the control animals to 15.59 in drug-treated animals. A marked pathogenetic difference was observed in drug-treated animals. Animals which died had massive amounts of virus antigen in the brain as determined by immunofluorescence. Untreated animals did not have virus antigen in the brain, detectable by this technique. This observation includes one untreated animal which died after a long incubation period (8 days) which overlapped with the incubation period of the drug-treated animals.

FOREWORD

In conducting the research described in this report, the investigator adhered to the *Guide for the Care and Use of Laboratory Animals,* prepared by the Committee on Care and Use of Laboratory Animal;s of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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A. PRIMARY TESTING: CONGO-CRIMEAN HEMORRHAGIC FEVER VIRUS (CCHF)

SUMMARY OF DRUGS TESTED

Fifty-five tests have been done measuring compounds for efficacy against CCHF (TABLE 1). The details of the z-score related to the virus rating (VR) is given in a later section. An additional 18 drugs have been tested for toxicity at 50 mg/kg and in many cases at lower levels and have been toxic at all levels tested, some as low at .25 mg/kg. The total number of drug tests is 73 (TABLE 1). Detailed data for each drug are in the APPENDIX.

DESCRIPTION OF THE MODEL AND ANALYSIS OF DATA

The CCHF model is as follows. Drugs have been tested for toxicity in infant mice (1-2 days old) at 50 mg/kg. Those which were not toxic were subjected to testing against CCHF, strain 10200, passage 11 in infant mouse brain tissue. Each drug was given to infant mice in a volume of 0.075 mls., ip. Forty-five minutes later, virus, in dilutions ranging from 1:100 to 1:1,000,000, was inoculated ip in a volume of 0.075 mls. Mock-treated mice were given tissue culture medium as control (DMEM). Mice were observed daily.

Geometric mean times to death were calculated for control mice (VC) and for drug-treated mice (VR). The geometric mean time to death (VC) is equal to the nth root (where n=total number of animals) of the product of each day with mortality raised to the power of the number of animals dying on that day. The geometric mean time to death (VR) for each drug is equal to the ratio of the geometric mean time to death for each drug divided by VC. As soon as a single positive drug was identified, that drug was incorporated into each test as a measure of test sensitivity.

A second direct measure of mortality was used as comparison with the VR. In this instance, a protective effect was calculated. This was determined by substracting the log LD_{50} virus titer in drug-treated animals from the log LD_{50} virus titer in mock-treated animals. A protective index of 1.7 means protection against 50 LD_{50} of virus, since the log (base 10) of 1.7 is 50. This index has been widely used for determination of neutralization antibody.

TEST REPRODUCIBILITY AND SENSITIVITY

Inherent variation in the test model was examined by determining VC's for several different experiments. After a single placebo dose over six different experiments, the mean VC (in days) was 12.05 with an SE of 0.66. After 3 multiple dose experiments, the mean VC was 10.03 with a SE of 0.73 (TABLE 2).

The sensitivity of the test system was monitored by inclusion of the positive drug in those tests which occurred after identification of this drug (AVS#1). In tests in which single doses

of drug were used, the range of VR+ values was 1.85 to 2.05 with a mean of 1.87 and a standard error of 0.13. In multiple drug tests, the mean was 2.78 with a standard error of 0.14 (TABLE 3). The fact that our positive control often had a VR less than two is dealt with in a later section.

ANALYSIS OF MORTALITY DATA (PROTECTIVE EFFECT, PE)

Drug effects measured by protective effect are an indicator of reduced mortality, irrespective of survival time. Most of the drugs tested had little or no significant effect on mortality from CCHF, as shown by the fact that the mode is in bar 1, PE's ranging from 0 to 0.23 (FIGURE 1). Notable exceptions occurred and resulted in the mean value being significantly higher than the mode. Drugs significantly affecting mortality (PE >1.61) are shown in bars 9 & 12. These data are influenced by the potency of one drug AVS#1 which was run repeatedly in our tests. In some of these tests the PE was significantly higher than the VR because time to death and mortality in this instance were apparently independent events.

ANALYSIS OF SURVIVAL TIME AND MORTALITY (VR)

The distribution of VR scores is given in FIGURE 2. Most of the drugs tested had some slight effect on survival time as shown by the fact that the mode is in bar 4 (VR, 1.15-1.27). In this distribution, the mode and median are closely juxtaposed further emphasizing the fact that most drugs had an effect on survival time. The mean (1.45) was in bar 6 (1.39-1.51) demonstrating the influence of those drugs which had a significant effect on prolonging survival time.

DETERMINATION OF SIGNIFICANCE

The next task was to determine the significance of the VR (virus rating) values. An arbitrary cutoff of 2 meant that AVS#1 and other drugs had no beneficial value. Clearly this was not true as determined by the significant protective effects observed. For example, although the VR was only 1.85, the protective effect was 2.59. This resulted because the drug decreased mortality, but did not, in this test, alter survival time for animals which died. To solve this type of problem, the data were subjected to further analysis.

Data were transformed into a normal curve and a standard form by use of z-scores. The skewed z-score distribution (FIGURE 3) shows that most drugs were of little beneficial effect ($z=-1$) and further that a few drugs had a pronounced positive effect on survival time ($z>0$). The z-distribution was used to determine which drugs were of beneficial value by determining individual z-scores, and the area under the normal curve which these scores represented. In this manner, drugs of significance could be selected for additional testing. This is a particularly useful system when many drugs testing show some slight effect on survival time.

To illustrate, drug AVS#1, in one test, had a VR of 1.85. The

z-score is 0.82. Its beneficial effect was greater than 79% of all the other drugs. Drug AVS#253 had a VR of 1.66, z-score of 0.43 and thus its beneficial effect was greater than 66% of all the drugs tested. In a second test AVS#1 had a VR of 2.05. Its z-score was 1.23; the beneficial effect was greater than 89% of the drugs tested. By these calculation, the most beneficial drugs can be selected based on positive z-score values. Z-scores and percentiles for all drugs tested are listed in TABLE 1.

COMPARISON OF PE VERSUS VR

Correlation between protective effect and VR is shown in FIGURE 4. The correlation coefficient is 0.76. The lack of more stringent correlation is because prolonged survival time and decreased mortality were not always absolutely linked, as in the example cited above. For reasons of consistency with the entire drug testing program, analysis of data is based on the measure of VR only with recommended significance determined by z-score.

ANALYSIS OF MULTIPLE DOSE DRUG TESTS

Selected drugs were tested for toxicity using multiple doses of drug. The schedule adopted included one dose at 50 mg/kg, followed by daily doses of 10 mg/kg for each of four succeeding days. Drugs selected for multiple dose testing included some which were of positive efficacy in single dose testing (AVS#1, refer to TABLE 3), one which was marginal (AVS#253), some which were not of efficacy in single dose testing (AVS#'s 52, 95, 332, 646, 1829, 1831).

In no instance did a drug, negative in single dose testing, become positive after multiple dose testing (TABLE 4). This observation confirms that beneficial drugs are not being missed by single dose testing.

Noteworthy is the fact that multiple mock injections shortened the survival time in control animals (Refer to TABLE 2). The potency of drug AVS#1 was increased after multiple doses (Refer to TABLE 3). These data are summarized in FIGURE 5 which shows a bar chart of protective effects, including AVS#1. The frequency distribution of z-scores emphasizes the relative potency of AVS#1. (FIGURE 6).

The conclusion from these data is that multiple dose tests are most useful when a compound has shown clear potency in the single dose test. Promising drugs have been recognized in recent single dose tests, and these will be subjected to multiple dose tests for confirmation.

B. PRIMARY TESTING: LYMPHOCYTIC CHORIOMENINGITIS VIRUS

One of the great disappointments of the first year's work on this contract has been the difficulties experienced with LCM virus in adult mice. The Bulgarian strain which was proposed for use was unique in that it killed adult mice after peripheral inoculation. At the time of initiation of this contract, the virus was preserved as

a 10% stock of infected infant mouse brain tissue. This stock was scarcely viable and had to be passed through adult mice to regain virulence. However, the first peripheral passages did not yield virus.

Therefore, the virus was passaged by intracerebral inoculation of adult mice. The adult mouse brain stock had a low peripheral titer, killing only half the inoculated mice at the highest concentration used. This stock was subsequently passaged twice by peripheral inoculation of infected spleen cells tissue. After two successive passages, a virus stock was prepared which had a peripheral titer sufficient for drug testing.

In the interim, virus from infected spleen tissue was plaqued in Vero cells and individual plaques were tested for peripheral virulence. Cloned stocks of virus have been prepared and are being tested for virulence.

Virus stocks are now available for large scale testing of drugs in the LCM model. While much time was lost trying to restore the virulence of the virus stock, that investment is now ready to pay dividends. The backlog of drugs which we have on hand should be finished within a short period. This seems the rational solution in view of the enormous resource expenditure which has already been expended on reestablishing the LCM mouse model. If unforseen problems develop in the mouse model, we would propose switching to a hamster model since this is the natural host for this virus.

C. SECONDARY TESTING: YELLOW FEVER VIRUS AND AVS#1 IN PRIMATES

DRUG TREATMENT AT THE TIME OF AND AFTER VIRUS EXPOSURE.

In the first experiment in squirrel monkeys, Saimiri sciureus, animals were inoculated subcutaneously with 1000 LD_{50}'s of yellow fever virus, Dakar 1279 strain, in the 8th infant mouse brain tissue passage. Drug AVS#1 was given subcutaneously at the time of virus inoculation and daily thereafter using doses of 50 mg/kg based on the published literature. No signs of drug toxicity were observed.

Drug treatment had no significant effect on mortality. One of two virus infected animals survived whereas 1 of 3 drug treated animals survived (TABLE 5).

The geometric mean time to death for the control animals was 9.2 days compared to 9.44 days for drug treated animals. The VR for the drug was 1.03 (TABLE 6).

The mean peak viremia level (day 3 after inoculation of virus) in control animals was 6.8 log PFU in contrast to 5.1 in treated animals. Surviving control animals had a slightly higher HI response, but NT antibody titers were comparable. Alkaline phosphatase levels were elevated in the control animals, but only on day 3. This change was not correlated with death. A single drug treated animal remained healthy and did not seroconvert (See

APPENDIX for data).

DRUG TREATMENT BEFORE AND AFTER VIRUS EXPOSURE

In the second experiment with squirrel monkeys, drug treatment (50 mg/kg) was initiated 3 days prior to virus exposure and continued for 8 days after virus injection. Virus and drug doses were similar to those described above. All animals used in this test were pre-screened by plaque reduction neutralizing antibody test in Vero cells (clone E6). Of fifteen animals screened, 12 were free of antibody which neutralized yellow fever virus. These data suggest a natural infection rate of 3/15 or 20%. One pre-screened animal died before purchase.

Drug treatment reduced mortality from 80% (5/6) in untreated controls to 40% (2/5) in the drug treated animals. When analyzed by Fisher's Exact Test, the difference had a p value of 0.20 (**TABLE** 5).

The geometric mean time to death for control animals was 6.1 days and for treated animals, 15.6 days. The VR was 2.54, suggesting that drug treatment prolonged survival time. However, independent T test analysis revealed a p value of 0.065 (**TABLE** 6).

Further statistical analysis by the Kolmogorov-Smirnov 2-sample test shows that to establish statistical significance for these data, a sample size of 21 would be needed, based upon proportions suggested in this test, with 5% beta error and 1% alpha error.

Daily temperatures were recorded for all animals; these data revealed no correlations with survival except for the fact that all sick animals had severely depressed body temperatures, perhaps as a result of lack of movement.

Other analyses have not been completed at this date. These include blood chemistries, viremia levels, and antibody determinations, and histopathology.

One very striking difference between control and treated animals was observed by immunofluorescent staining of tissues from moribund animals. In both control and drug treated animals, yellow fever virus antigen was detected, using fluorescein-labeled antibody in hepatocytes, splenocytes, and in epithelial cells of the kidney glomerulus (**FIGURE** 7A & B). Infection in hepatocytes was most widespread whereas antigen in other tissues was focal and less predominant. Viral antigen was not detected in the central nervous system, either in neural or endothelial cells, in untreated animals.

In contrast, the central nervous system of drug-treated animals was heavily infected with yellow fever virus as determined by immunofluorescence. Antigen was widespread in the endothelial cells of brain capillaries (**FIGURE** 7C &D), in neuronal cells (**FIGURE** 7E) , and in smaller neural cells. Virus antigen could be found in neuronal cell bodies and in the processes projecting from the cell body as these processes transversed brain tissue (**FIGURE** 7F). Thus, axoplasmic transport could account, at least in part, for the

widespread infection of the nervous system. There was evidence of hemorrhaging in the substance of the brain tissue. Histopathologic examination of the tissues is required to confirm this finding; this is in progress as are virus isolations.

Although our findings are based on upon immunofluorescence, the data suggest that animals administered drug died of a syndrome different from that of untreated animals. This hypothesis is strengthened by the fact that an untreated animal died at 8 days, the same general time frame as the treated animals, without signs of central nervous system involvement.

One possible explanation for the altered pathogenesis is that the drug changes the the permeability of blood-brain barrier as physiologic changes have been shown to do. Alternatively, incorporation of the drug into the virion might alter its tropism for central nervous system tissue. How such a change might occur remains speculative. Completion of our ongoing studies may contribute to an explanation for this observation.

TABLE 1

SUMMARY OF RESULTS OF DRUG TESTING IN THE CCHF MODEL

A. Virus-Drug Tests

AVS #	Drug dose	Protective effect	Virus Rating VR	VR+	Z	Percentile(%)
1	**S**	**2.59**	**1.85**	**1.85**	**0.82**	**79**
1	**S**	**2.70**	**2.05**	**2.05**	**1.23**	**89**
1	**S**	**ND**	**2.39**	**2.39**	**1.94**	**97**
1	**M**	**4.20**	**2.88**	**2.88**	**2.96**	**99**
1	**M**	**3.60**	**2.68**	**2.68**	**2.54**	**99**
1	**S**	**2.0**	**1.73**	**1.73**	**.57**	**72**
1	**S**	**2.0**	**1.85**	**1.85**	**.82**	**79**
33	**S**	**ND**	**1.75**	**2.39**	**.61**	**73**
52	S	1.00	1.03	ND	-.88	19
52	M	0.30	0.87	2.88	-.12	11
54	S	1.20	1.32	ND	-.28	39
64	S	0.40	1.28	2.05	-.36	36
68	S	0.20	1.09	2.05	-.76	22
70	S	0.60	1.24	ND	-.45	33
71	**S**	**ND**	**2.39**	**2.39**	**1.94**	**97**
78	**S**	**ND**	**2.39**	**2.39**	**1.94**	**97**
79	S	0.90	1.39	1.73	-.13	45
84	S	0.65	0.98	ND	-.99	16
87	S	0	1.09	1.85	-.76	22
94	S	0.3	1.18	2.05	-.57	28
95	S	0.9	1.24	ND	-.45	33
111	S	0	1.13	ND	-.67	25
113	S	0.5	1.20	2.05	-.53	30

TABLE 1 CONT.

A. Virus-Drug Tests

AVS #	Drug dose	Protective effect	Virus Rating VR	VR+	Z	Percentile(%)
181	S	0	0.97	ND	-1.01	16
200	S	0.7	1.66	ND	.43	66
215	S	0.5	1.33	ND	-.26	40
253	S	1.41	1.44	1.85	-0.3	49
253	M	1.3	1.68	2.88	.47	68
257	S	0.3	1.18	1.85	-.57	28
272	S	0.7	1.23	ND	-.47	32
332	S	0.9	1.26	ND	-.40	34
332	S	0	1.24	1.85	-.45	33
345	S	0.2	1.22	ND	-.49	31
346	S	0	1.11	ND	-.72	24
349	S	0	0.88	ND	-1.19	12
646	S	0.97	1.25	1.85	-.43	34
646	S	0.5	0.96	1.85	-1.03	15
701	S	ND	0.85	2.39	-1.26	10
1199	S	0	0.97	2.05	-1.01	16
1199	S	0.2	1.14	1.73	-.65	26
1829	S	0.4	1.04	ND	-.86	19
1829	M	0.2	0.84	2.68	-1.28	10
1831	S	0.5	1.01	ND	-.92	18
1831	M	0	1.09	2.68	-.76	22
1834	S	0.2	1.21	2.05	-.51	31
1846	M	0	0.99	2.68	-.96	17
1915	**S**	**ND**	**2.01**	**2.39**	**1.15**	**88**
1968	**S**	**ND**	**2.11**	**2.39**	**1.36**	**91**
1970	**S**	**ND**	**2.39**	**2.39**	**1.94**	**97**
1977	**S**	**ND**	**2.11**	**2.39**	**1.37**	**99**
1978	S	ND	1.55	2.39	0.2	58
1986	S	ND	1.58	2.39	0.26	60

TABLE 1 CONT.

A. Virus-Drug Tests

AVS #	Drug dose	Protective effective	Virus Rating VR	VR+	Z	Percentile(%)
1989	S	ND	2.04	1.21	1.21	89
1988	S	ND	1.85	2.39	.82	79
2023	S	ND	2.39	2.39	1.94	97
2137	S	ND	2.39	2.39	1.94	97

B. REMAINING TOXICITY PROBLEMS

AVS #	Toxic at Drug Dose mg/kg
148	0.25
70,1841,1901	10
136,347,360,361,593,1089,1160	50
1843,1986,1990,2138,2139,2140,	50
2159,2160	50

C. SUMMARY OF DRUG TESTED IN CCHF MODEL

Total Testing Completed	Additional Toxicity Tests Required	Total
56	19	75

TABLE 2

TEST VARIATION IN CCHF MODEL
GEOMETRIC MEAN TIME
TO DEATH (VC) FOR CCHF VIRUS CONTROL*

Single Placebo Dose	Multiple Placebo Dose
11.90	10.45
11.75	9.19
12.98	10.45
12.79	-
11.10	-
12.20	-
Mean ± SE 12.05 ± 0.66	10.03 ± .73

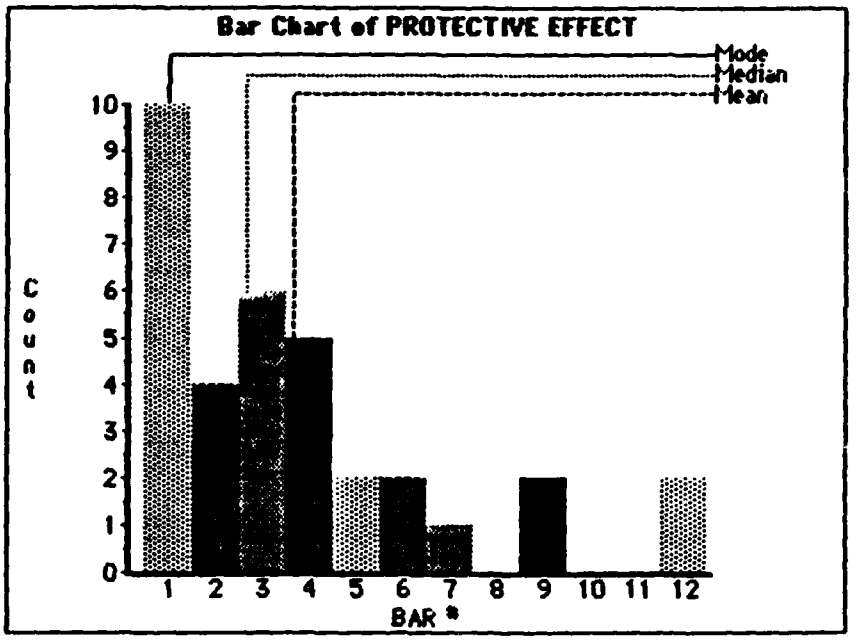
On this and subsequent tables:
*Strain 10200, i.p., 0.075 ml in log dilutions 1:100 to 1:1000,000
In a single dose study, virus was inoculated 45 min before placebo (DMEM) or drug (0.075ml, ip).
In a multiple dose study, additional inoculations were given on days 1 to 4, 10mg/kg.
Vc is equal to the n root (n=total number of animals) of the product of each day with mortality raised to the power of the number of animals dying on that day.

TABLE 3

GEOMETRIC MEAN TIME TO DEATH
FOR A POSITIVE CONTROL (AVS#1,VR+)

Single Drug Dose	Multiple Drug Dose
1.85	2.88
2.05	2.68
1.73	-
1.85	-
Mean ± SE 1.87 ± 0.13	2.78 ± 0.14

FIGURE 1



PROTECTIVE EFFECT

Bar:	From: (≥)	To: (<)	Count:	Percent:	
1	0	.23	10	29.412	←Mode
2	.23	.46	4	11.765	
3	.46	.69	6	17.647	
4	.69	.92	5	14.706	
5	.92	1.15	2	5.882	
6	1.15	1.38	2	5.882	
7	1.38	1.61	1	2.941	
8	1.61	1.84	0	0	
9	1.84	2.07	2	5.882	
10	2.07	2.3	0	0	
11	2.3	2.53	0	0	
12	2.53	2.76	2	5.882	

FIGURE 2

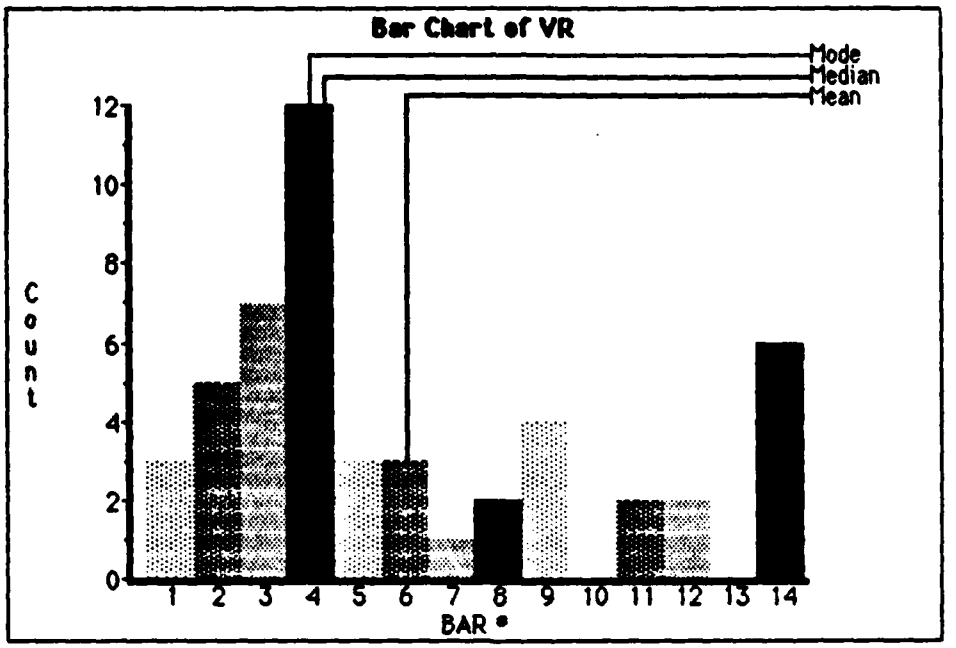

Bar Chart of VR
Mode
Median
Mean
12
10
8
6
4
2
0
Count
1 2 3 4 5 6 7 8 9 10 11 12 13 14
BAR #

FIGURE 3

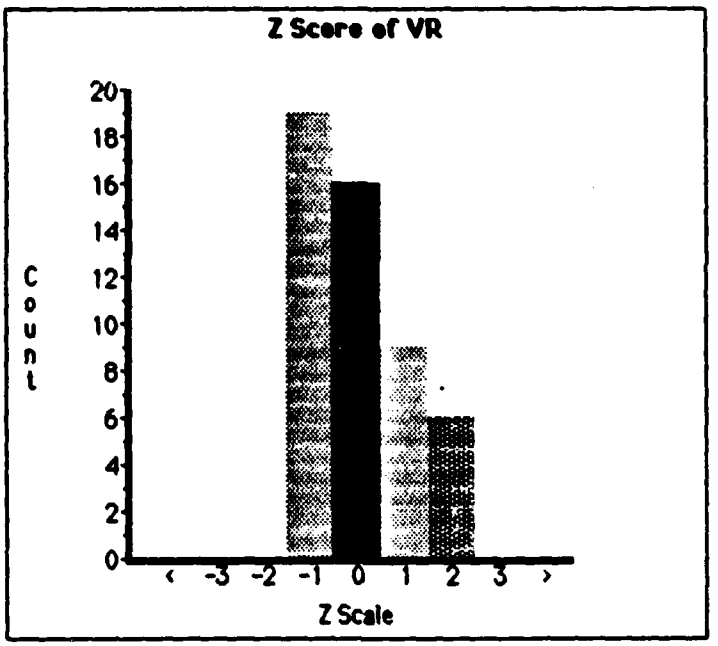


FIGURE 4

PE VERSUS VR

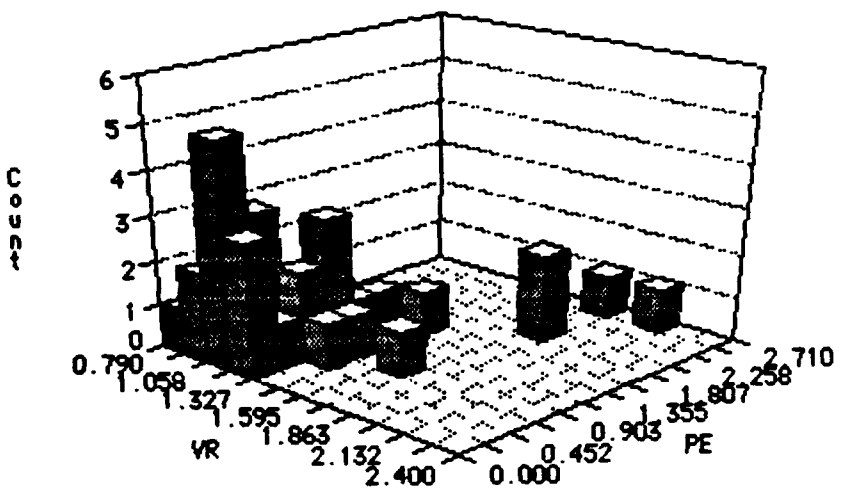


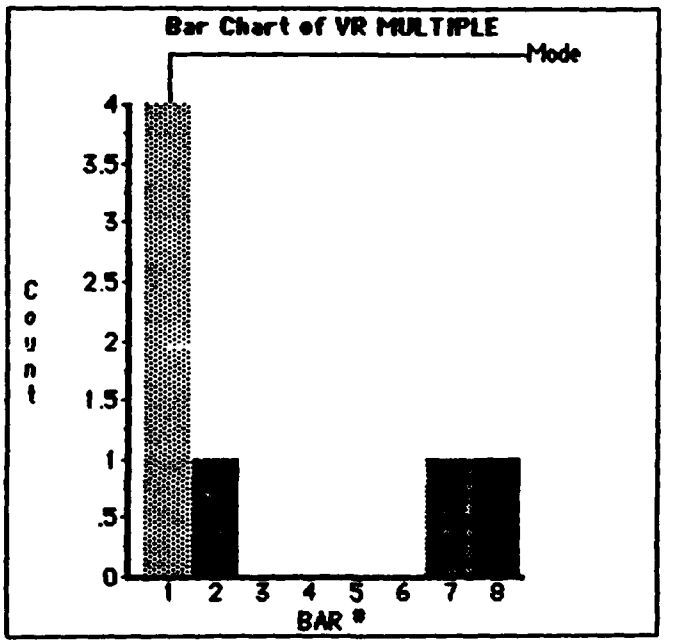
TABLE 4

MULTIPLE TESTS IN THE CCHF MODEL WITH SELECTED DRUGS*

AVS #	Drug Dose	Protective Effect	VR	VR+
52	Single	1.00	1.03	ND
52	Multiple	0.30	0.87	2.88
95	Single	0.90	1.24	ND
95	Single	1.3	0.79	1.85
253	Single	1.41	1.44	1.85
253	Multiple	1.3	1.68	2.88
332	Single	0.9	1.26	ND
332	Single	0	1.24	1.85
646	Single	0.5	0.96	1.85
646	Single	0	0.97	2.05
1829	Single	0.4	1.04	ND
1829	Multiple	0.2	0.84	2.68
1831	Single	0.5	1.01	ND
1831	Multiple	0	1.09	2.68

On this and subsequent tables:
*The Protective Effect is the difference in log titer between virus alone and virus in drug treated animals.
VR+ is the VR of drug AVS #1.

FIGURE 5



VR MULTIPLE

Bar:	From: (≥)	To: (<)	Count:	Percent:	
1	.84	1.13	4	57.143	←Mode
2	1.13	1.42	1	14.286	
3	1.42	1.71	0	0	
4	1.71	2	0	0	
5	2	2.29	0	0	
6	2.29	2.58	0	0	
7	2.58	2.87	1	14.286	
8	2.87	3.16	1	14.286	

FIGURE 6

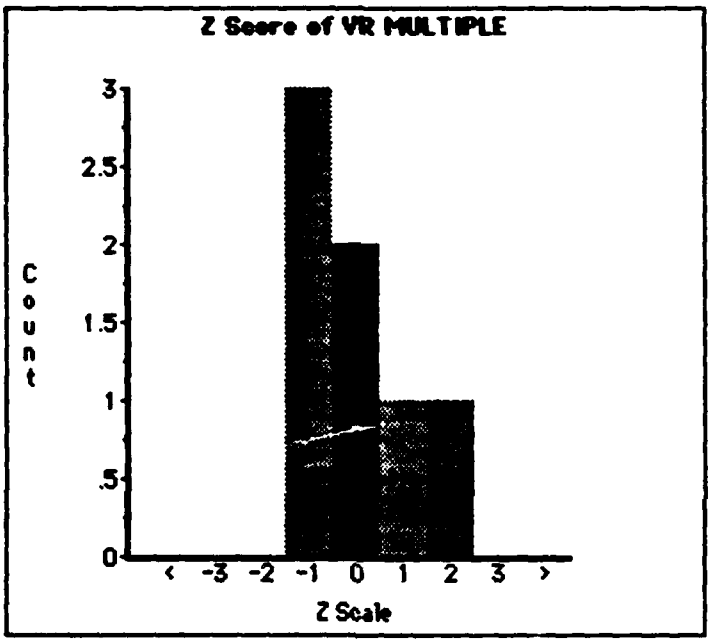


TABLE 5

SUMMARY OF SECONDARY TESTING OF AVS DRUG # ONE IN YELLOW FEVER PRIMATE MODEL

	Mortality	
	Virus and Drug	Virus Alone
Treatment after Virus Exposure	2/3 (67%)	1/2 (50%)
Treatment before and after Virus Exposure*	2/5 (40%)	5/6 (80%)

*P=0.20 by Fisher's Exact Test.

TABLE 6

GEOMETRIC MEAN TIME TO DEATH (VR) IN THE YELLOW FEVER PRIMATE MODEL

Experiment #1 (Drug #1 after Virus)

Virus		Virus and Drug	
DAYS	#Dead	DAYS	#Dead
3	1	5	1
28	1	6	1
		28	1

V(control)=9.17

V (drug)=9.44

VR=1.03

Experiment 2 (Drug #1 Before and After Virus)

Virus		Virus and Drug	
DAYS	#Dead	DAYS	#Dead
3	1	6	1
4	2	7	1
5	1	28	3
8	1		
28	1		

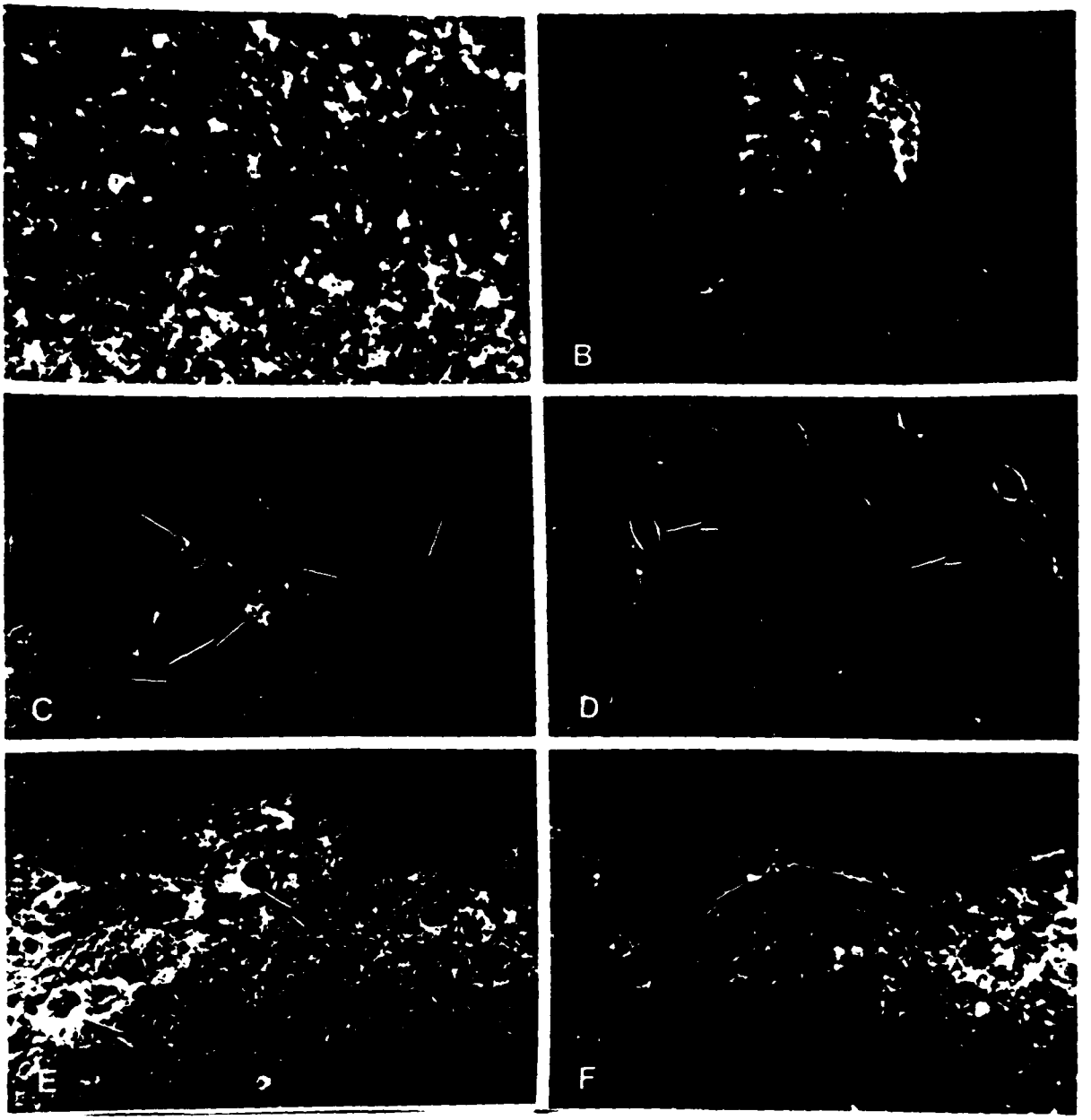
V(control)=6.14

V(drug)=15.59

VR=2.54

P=0.065

FIGURE 7. LOCALIZATION OF VIRUS ANTIGEN BY IMMUNOFLUORESCENCE.



FIGURES A-B are impression sections from liver and kidney of an untreated animal 5 days after yellow fever virus inoculation. **FIGURES C-F** are impression brain sections from a treated animal 8 days after virus inoculation. Impressions were incubated with mouse ascitic fluid to yellow fever virus (SV45) and goat anti-mouse FITC-labeled second antibody. **A.** Widespread involvment of hepatocytes. **B.** Focal localization in the glomerulus. **C.** Specific fluorescence in the cytoplasm of capillary endothelial cells (arrowheads) and in the cell body of an adjacent neuron (arrow). **D.** Higher magnification of fluorescence in capillary endothelial cells (arrows). **E.** Heavily stained Purkinje and granule cells in the cerebellum **F.** A large neuron in the cortex with fluorescence in the cell body (arrow) and in an axon.

TABLE 7

DISTRIBUTION OF YELLOW FEVER VIRUS ANTIGEN IN PRIMATES BY IMMUNOFLUORESCENCE

Animal Number	91	92	88	94	89
Experimental condition	Virus	Virus	Virus	Virus + drug	Virus
Day of death (pi)	4	4	5	7	8
Organ(1)					
Liver	++(2)	+++	+++	+++	+++
Spleen	+	++	++	++	++
Brain (neurons)	-	-	-	++++	-
Kidney	+	++	ND	ND	ND

(1) Impression of primate hearts and lungs were also processed; these were uniformly negative.

(2) Approximate percentage of fluorescent cells in impression sections (+ -25%, ++ 50%, +++ 75%, ++++ 100%) after incubation with yellow fever virus antibody and FITC-labeled second antibody. Control slides were incubated with unrelated antibody and FITC-labeled second antibody. Fluorescence on control sections was not observed.

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APPENDIX

DEVELOPMENT AND TESTING OF ANTI-VIRAL DRUGS
SECONDARY TESTING, PRIMATE MODEL
YALE ARBOVIRUS RESEARCH UNIT

FINAL REPORT

DATE OF REPORT: 10/24/86

TOXICITY TEST: SECONDARY, PRIMATE DATE OF TESTING: 10/3/86

Drug number:	1	Animal species:	Saimiri sciureus
Drug toxicity:	50 mg/kg	Route of inoculation:	SC
MTC:	>50 mg/kg	Age/wt of animal:	Adult; 526 gms

Animal identification number and weight: #3; 526 gms

Other treatments given: Anesthesia Bled (1.5 mls), Days 3-7,14

Dose: 20 MG Ketamine & 0.1 mg Atropine

Route of administration: SC

EXPERIMENTAL PROCEDURE FOR VIRUS-DRUG TEST

DATE OF VIRUS-DRUG TESTS: 10/3/86

Virus: Yellow fever, strain Dakar 1279 passage 8, infant mouse brain tissu

Virus dose & route: 1000 LD_{50}, SC

Number of animals given virus & drug: 3 Animal identity: #2,#4,#5

Drug dose: 50 mg/kg DAILY

Antibody titers (Pre): HI: All <1:10, except #3: 1:20 (NT not yet done)

Number of animals given drug only: 1 Animal identity: #3 (see above)

Number of animals given virus only: 2 Animal identity: #1 & #6

Animal number & weight (Virus only): #1, 491 gms
#6, 519 gms

Number of animals given virus & drug: 3 Animal identity: #2, #4, #5

Animal number & weight (Virus & drug): #2, 593 gms
#4, 430 gms
#5, 499 gms

DEVELOPMENT AND TESTING OF ANTI-VIRAL DRUGS
SECONDARY TESTING, PRIMATE MODEL
YALE ARBOVIRUS RESEARCH UNIT

EXPERIMENTAL RESULTS

Mortality (Virus & drug):	2 of 3 inoculated (#4 & #5)	
Mortality (Virus only):	1 of 2 inoculated (#1)	
Mortality by date (Virus & drug):	#4, D6 (10/9/86) #5, D5 (10/8/86)	
Mortality by date (Virus only):	#1, D3 (10/6/86)	
Date of onset of illness (Virus & drug):	D5, D6	
Date of onset of illness (Virus only):	D3	
Survival (Drug only):	1 of 1 tested (#3)	
Survival (Virus & drug):	1 of 3 inoculated (#2)	
Survival (Virus only):	1 of 2 inoculated (#6)	
Necropsy findings (Virus & drug):	Antigen in liver by FA #4	
Necropsy findings (Virus only):	Antigen in liver by FA #1	
Day of onset of viremia (Virus & drug):	D3 (10/6/86) for #4 & #5 (#2 neg)	
Day of onset of viremia (Virus only):	D3 (10/6/86) for #1 (#6 neg)	
Days viremic (Virus & drug):	One day: D3 (Not bled D1 & D2)	
Days viremic (Virus only):	One day: D3 (Not bled D1 & D2)	
Viremia titers (Virus & drug): (in Vero Cells)	#4, 6.1 log PFU/ml (Confirmed by ELISA) #5, 4.2 log PFU/ml (Confirmed by ELISA)	
Viremia titers (Virus only): (in Vero Cells)	#1, 6.8 log PFU/ml	
Antibody titers (Virus & drug):	HI: #2, 1:10 on D10 #3, drug only, 1:20 NT: #2 >1:40 on D10 NT: #3, drug only, 1:40	
Antibody titers (Virus only):	HI: #6, 1:40 on D10 NT: #6, >1:40 on D10	
Blood chemistry:		
Alkaline phosphatase:	Elevated in #1 & #6 (Virus only) on D3	

COMPUTER PROGRAM FOR CALCULATION OF VR SCORES

```
10    REM    ------------------------------------------------------
20  REM                VR CALCULATIONS
30    REM    ------------------------------------------------------
40  REM                                                        ric
50    REM    ------------------------------------------------------
55  CLEAR
60  PRINT "MASTER MENU OPTIONS:"
70  PRINT  "====================":PRINT
75   PRINT "  1) VR Calculations using the printer"
80   PRINT "  2) VR Calculations without the use of a printer"
90   PRINT "  3) PFU Calculations
92  PRINT " 4) REED and MUENCH
95   PRINT "  5) QUIT"
100  PRINT " SELECT THE NUMBER OF YOUR CHOICE"
110   INPUT R
120   IF R=1 THEN GOTO 202
130   IF R=2 THEN GOTO 900
132   IF R=3 THEN GOTO 1360
133   IF R=4 THEN GOTO 2000
134   IF R=5 THEN GOTO 3000
200   PRINT "INVALID INPUT"
201   GOTO 100
202   CLEAR:LPRINT
203  INPUT "ENTER VIRUS AND STRAIN";V$
204   PRINT TAB(5)  V$
205   LPRINT TAB(30)  V$
212  INPUT "ENTER DATE(MMDDYY)";A$
213   PRINT A$
214   LPRINT TAB(5)">>>DATE>>> ";A$:LPRINT
216   INPUT "ENTER LD50" ;B$
217   PRINT B$
218   LPRINT TAB(5)">>>LD50>>> ";B$
219   LPRINT
220   PRINT TAB(30) "VR-DRUG CALCULATIONS"
225   PRINT TAB(30) "====================="
230   LPRINT TAB(30) "VR-DRUG CALCULATIONS"
240    LPRINT TAB(30)   "********************"
250   LPRINT
256   LPRINT
260   LPRINT
261   LPRINT
290   PRINT
295   PRINT "ENTER CONTROL DATA":PRINT
300   PRINT "NUMBER OF EXPERIMENTS =  ";
305   PRINT
```

```
310  INPUT Z
320  FOR T=1 TO Z
330  IF T=1 THEN 340 ELSE 346
340  LPRINT TAB(30) "CONTROL CALCULATIONS"
345  LPRINT TAB(30) "======================":LPRINT:GOTO 380
346  PRINT "ENTER EXPERIMENTA DATA :":PRINT
350  PRINT "ENTER AVS NUMBER ";
360  INPUT AVS
365  PRINT
370  LPRINT TAB(30) "EXPERIMENTAL CALCULATIONS AVS NO.";AVS
375  LPRINT TAB(30) "==================================":LPRINT
380  M=0
390  P=0
400  A=0
410  B=0
420  C=0
430  G=0
440  L=0
450  PRINT "NUMBER OF DAYS =  ";
455  PRINT
460  INPUT N
470  FOR I=1 TO N
480  PRINT "ENTER DAY,ACCUMULATED DEATHS";I;
490  INPUT A,B
500  IF I=1 THEN 510 ELSE 520
510  GOSUB 800
520  LPRINT TAB(30) A;TAB(45) B
530  P=M+B*LOG(A)
540  M=P
550  C=C+B
560  L=M/C
570  LPRINT
580  NEXT I
581  LPRINT
582  G=EXP(L)
610  IF T=1 THEN V=G ELSE X=G
625  PRINT
635  PRINT TAB(30) "X=   ";X
640  LPRINT USING "                    X= ##.#";X
645  PRINT TAB(30) "V=    ";V
650  LPRINT USING "                     V= ##.#";V
655  PRINT TAB(30) "VR=    ";X/V
660  LPRINT USING "                     VR=##.#";X/V
670  LPRINT
680  LPRINT
```

```
690   NEXT T
700   PRINT "MORE DATA? (YES=1,NO=0)"
710   INPUT W
720   IF W=1 THEN 202 ELSE 55
800   LPRINT TAB(30) "DAYS";TAB(45) "DEATHS"
810    LPRINT TAB(30)  "----";TAB(45)  "------"
820   RETURN
822   CLEAR
900   PRINT TAB(30) "VR-DRUG CALCULATIONS"
910   PRINT TAB(30) "====================="
920    PRINT
930   PRINT "ENTER CONTROL DATA":PRINT
940   PRINT "NUMBER OF EXPERIMENTS =  ";
950    PRINT
960    INPUT Z
970    FOR T=1 TO Z
980    IF T=1 THEN GOTO 1030
990   PRINT "ENTER EXPERIMENTAL DATA:":PRINT
1000    PRINT "ENTER AVS NUMBER ";
1010    INPUT AVS
1020    PRINT
1030    M=0
1040    P=0
1050    A=0
1060    B=0
1070    C=0
1080    G=0
1090    L=0
1100   PRINT "NUMBER OF DAYS=  ";
1110    PRINT
1120    INPUT N
1130    FOR I=1 TO N
1140   PRINT "ENTER DAY, ACCUMULATED DEATHS";I;
1150     INPUT A,B
1180    P=M+B*LOG(A)
1190    M=P
1200    C=C+B
1210    L=M/C
1215    NEXT I
1230    G=EXP(L)
1240    IF T=1 THEN V=G ELSE X=G
1250    PRINT
1260    PRINT TAB(30) "X=    ";X
1270    PRINT TAB(30) "V=    ";V
1280    PRINT TAB(30) "VR=   ";X/V
```

```
1290  PRINT
1300  NEXT T
1310  PRINT "MORE DATA? (YES=1, NO=0)"
1320  INPUT VT
1330  IF VT=1 THEN 822 ELSE 55
1360  PRINT "=================================================="
1370  PRINT "                    PFU CALCULATIONS                "
1380  PRINT "=================================================ric===="
1390  PRINT
1395  CLEAR
1400  DIM D(30,30)
1410  INPUT "ENTER THE AMOUNT OF INOCULUM ";R
1420  PRINT
1430  PRINT "ENTER NUMBER OF DILUTIONS";
1440  PRINT
1450  INPUT M
1460  PRINT
1470  FOR C=1 TO M
1480  INPUT "ENTER DILUTION  ";ED(C)
1490  PRINT
1500  NEXT C
1510  PRINT "ENTER THE NO. OF PLATES PER DILUTION";
1520  INPUT N
1530  PRINT
1540  PRINT "ENTER NO. OF PLAQUES BY DILUTION AND PLATE NO."
1550  FOR I=1 TO M
1560  FOR J=1 TO N
1570  INPUT D(I,J)
1580  NEXT J
1590  NEXT I
1600  PRINT
1610  FOR I=1 TO M
1620  FOR J= 1 TO N
1630  LET S=S+D(I,J)
1640  NEXT J
1650  NEXT I
1660  PRINT " SUM OF PLAQUES=";S
1670  PRINT "================"
1680  PRINT
1690  FOR C=1 TO M
1700  LET T=T+1/(10^ED(C))*N
1710  NEXT C
1720  PRINT
1730   X=S/T
1735  NL=LOG(X)
```

```
1736   CL=NL*.4343
1737  PRINT USING "COMMON LOG / INOCULUM=##.##";CL
1738  PRINT          "============================="
1739   PRINT
1740  PRINT USING "PFU= ##.###^^^^ PER INOCULUM";X
1750  PRINT          "============================="
1760   PRINT
1770  PRINT USING "PFU= ##.###^^^^ PER ML";1/R*X
1780  PRINT          "====================="
1790   PRINT
1800   SX=SQR(X/T)
1810  PRINT USING "SD= +/-.###^^^^";SX
1820  PRINT           "============="
1830   PRINT
1840    CV=(SX/X)*100
1850  PRINT USING "CV= ###.#%";CV
1860  PRINT          "==========="
1870   PRINT
1880  PRINT "MORE DATA? (YES=1, NO=0)"
1890   INPUT W
1900   IF W=1 THEN 1395 ELSE 55
2000  PRINT "=================================================="
2010   PRINT "                        REED and MUENCH                    ric          "
2020  PRINT "=================================================="
2025   CLEAR
2030   DIM  DA(12),S(12),SD(12),SS(12),P(12),DL(12)
2040  INPUT "NUMBER OF DILUTIONS= ";N
2050  FOR Z=1 TO N
2055  INPUT "DILUTION";DL(Z)
2060  NEXT Z
2070  FOR I=1 TO N
2080  INPUT "DEATHS";DA(I)
2090  NEXT I
2100  FOR J=1 TO N
2110  INPUT "SURVIVORS =";S(J)
2120  NEXT J
2130   SD(8)=D(8)
2140   SD(7)=SD(8)+DA(7)
2150   SD(6)=SD(7)+DA(6)
2160   SD(5)=SD(6)+DA(5)
2170   SD(4)=SD(5)+DA(4)
2180   SD(3)=SD(4)+DA(3)
2190   SD(2)=SD(3)+DA(2)
2200   SD(1)=SD(2)+DA(1)
2210   PRINT: PRINT
```

```
2220    SS(1)=S(1)
2230    SS(2)=SS(1)+S(2)
2240    SS(3)=SS(2)+S(3)
2250    SS(4)=SS(3)+S(4)
2260    SS(5)=SS(4)+S(5)
2270    SS(6)=SS(5)+S(6)
2280    SS(7)=SS(6)+S(7)
2290    SS(8)=SS(7)+S(8)
2300      P(1)=(SD(1)/(SD(1)+SS(1)))*100
2310      P(2)=(SD(2)/(SD(2)+SS(2)))*100
2320      P(3)=(SD(3)/(SD(3)+SS(3)))*100
2330      P(4)=(SD(4)/(SD(4)+SS(4)))*100
2340      P(5)=(SD(5)/(SD(5)+SS(5)))*100
2350      P(6)=(SD(6)/(SD(6)+SS(6)))*100
2360      P(7)=(SD(7)/(SD(7)+SS(7)))*100
2370      P(8)=(SD(8)/(SD(8)+SS(8)))*100
2380   LPRINT: LPRINT: LPRINT
2390   LPRINT "=========================================================
=============================="
2400   LPRINT "                          REED AND MUENCH
                                    "
2410   LPRINT "=============================================================ric======
=============================="
2420   LPRINT TAB(1)"DIL";TAB(10)"DEATHS";TAB(20)"SURVIVORS";TAB(35)"ACC DEA
THS";TAB(50)"ACC SURVIVORS";TAB(65)"CUM %";
2430   FOR A=1 TO N
2440   LPRINT TAB(1)DL(A);TAB(10)DA(A);TAB(20)S(A);TAB(35)SD(A);TAB(50)SS(A)
;TAB(65)P(A)
2450   NEXT A
2460   LPRINT: LPRINT
2470   IF P(8)>50 THEN 2480 ELSE 2490
2480   R=P(8):Y=DL(8):GOTO 2630
2490   IF P(7)>50 THEN 2500 ELSE 2510
2500   R=P(7):Y=DL(7):GOTO 2630
2510   IF P(6)>50 THEN 2520 ELSE 2530
2520   R=P(6):Y=DL(6):GOTO 2630
2530   IF P(5)>50 THEN 2540 ELSE 2550
2540   R=P(5):Y=DL(5):GOTO 2630
2550   IF P(4)>50 THEN 2560 ELSE 2570
2560   R=P(4):Y=DL(4):GOTO 2630
2570   IF P(3)>50 THEN 2580 ELSE 2590
2580   R=P(3):Y=DL(3):GOTO 2630
2590   IF P(2)>50 THEN 2600 ELSE 2610
2600   R=P(2):Y=DL(2):GOTO 2630
2610   IF P(1)>50 THEN 2620 ELSE 10
```

```
2620  R=P(1):Y=DL(1):GOTO 2630
2630  PRINT: PRINT: PRINT "R= ";R:PRINT "Y= ";Y
2640   M=Y/Y*10^-Y
2650  IF P(1)<50 THEN 2660 ELSE 2670
2660  T=P(1):GOTO 2820
2670  IF P(2)<50 THEN 2680 ELSE 2690
2680  T=P(2):GOTO 2820
2690  IF P(3)<50 THEN 2700 ELSE 2710
2700  T=P(3):GOTO 2820
2710  IF P(4)<50 THEN 2720 ELSE 2730
2720  T=P(4):GOTO 2820
2730  IF P(5)<50 THEN 2740 ELSE 2750
2740  T=P(5):GOTO 2820
2750  IF P(6)<50 THEN 2760 ELSE 2770
2760  T=P(6):GOTO 2820
2770  IF P(7)<50 THEN 2780 ELSE 2790
2780  T=P(7):GOTO 2820
2790  IF P(8)<50 THEN 2800 ELSE 10
2800  T=P(8):GOTO 2820
2810  LPRINT
2820  PRINT "T= ";T
2830  REM (% mortality next above 50%)-50% divided by
2840  REM (% mortality next above 50%)-(% mortality next below 50%)=
2850  REM proportionate distance
2860    PD=(R-50)/(R-T)
2870  LPRINT: LPRINT
2880  LPRINT "Proportionate distance = ";PD
2890  LPRINT
2900   C=(-1)*(Y+PD):LPRINT "C= ";C
2910   TI=10^C
2911  LPRINT
2912  LPRINT "TITER(decimal)= ";TI
2914  LPRINT
2915  CA=ABS(C)
2916  LPRINT USING "50% ENDPOINT DILUTION (LD50 TITRE)= 10^-#.#";CA
2930  PRINT "MORE DATA (YES=1, NO=0)"
2935   INPUT Z
2940  IF Z=1 THEN 2025 ELSE 55
3000  END
```

EXAMPLES OF COMPUTER OUTPUT OF VR SCORES

DATE
DURATION

VR-DRUG CALCULATIONS

CONTROL CALCULATIONS

DAYS	DEATHS
7	1
8	4

EXPERIMENTAL CALCULATIONS AVG NO. 1

DAYS	DEATHS
8	1
11	1
12	1
28	5